

# Osteoblast-related Transcription Factors Runx2 (Cbfa1/AML3) and MSX2 Mediate the Expression of Bone Sialoprotein in Human Metastatic Breast Cancer Cells<sup>1</sup>

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## ABSTRACT

Human breast cancers are known to preferentially metastasize to skeletal sites, however, the mechanisms that mediate the skeletal preference (orthotropism) of specific types of cancers remains poorly understood. There is a significant clinical correlation between the expression of bone sialoprotein (BSP) and skeletal metastasis of breast cancers. Our laboratory, as well as others, have proposed the concept that skeletal selective metastasis and associated disease may be attributable to a mimicry of skeletal cellular phenotypes by metastasizing cancer cells. We hypothesize that breast cancer cell expression of phenotypic properties of skeletal cell types, including BSP as one component of that phenotype, is the result of ectopic expression or activity of one or more central transcriptional regulators of bone cell gene expression. To test this hypothesis, we examined the molecular mechanisms that regulate *bsp* expression in human breast cancer cell lines with previously characterized metastatic potentials. Our results demonstrate that the majority of the distal *bsp* promoter sequences act to repress BSP expression in cancer cells and that most of the promoter activity resides in the proximal –110 bp of the *bsp* promoter. In this region, we identified a putative Runx binding element providing a basis for a mechanism for skeletal gene activation. Our results demonstrate that Runx2 is ectopically expressed in breast cancer cells and that one isoform of Runx2 can activate *bsp* expression in these cells. In addition, we observe that *bsp* expression is additionally regulated by the homeodomain factor *Msx2*, another regulator of osteoblast-associated genes. Thus, this is the first report of osteoblast-related transcription factors being expressed in human breast cancer cells and provides a component of a mechanism that may explain the osteoblastic phenotype of human breast cancer cells that preferentially metastasize to bone.

## INTRODUCTION

Death from cancer is usually attributable to the development of metastases, and the skeleton is the organ most frequently involved. Several cancers, including carcinoma of the breast, demonstrate a metastatic preference for bone in a phenomenon that involves osteotropism. Although it has been widely recognized that certain types of cancers display a propensity to metastasize preferentially to bone, the mechanisms directing this preferential localization and tissue integration are poorly understood.

The expression of *bsp*<sup>3</sup>, a gene normally associated with mineralization in skeletal cell biology, has been widely reported to correlate with the occurrence of skeletal metastases in several cancer types, including breast, prostate, and lung (1–9). Immunohistochemical evaluation of BSP expression in malignant breast

lesions revealed increased expression levels compared with benign lesions or normal breast tissue (4, 7). Elevated BSP expression was found to be a poor prognostic sign when present in the primary lesion, and there was a significantly increased incidence in the subsequent occurrence of bone metastasis in patients who expressed higher levels of BSP in the tumor tissue (2). It has also been recently reported that bone metastases express increased levels of BSP compared with visceral metastases from breast cancers (7). Taken together, these published reports demonstrate a strong clinical correlation between the expression of BSP and the preferential metastasis of breast carcinoma to bone.

Although relatively little is known about the functional role of BSP in breast cancer, its demonstrated association with mineralization in skeletal tissues suggests BSP may play a role in the microcalcifications commonly associated with primary breast lesions. More recently, additional functional activities have been associated with BSP, including the ability to bind factor H and protect cells from complement-mediated cellular lysis (10). In addition it has been demonstrated that BSP mediates human umbilical vein endothelial cell attachment and migration, thereby demonstrating an angiogenic capacity (11). Thus, BSP expression in metastatic breast cancers may provide enhanced angiogenic potential to metastasizing cancer cells in addition to the ability to evade complement-mediated lysis.

We hypothesized that the ectopic expression of the BSP by the breast cancer cells could be the consequence of the aberrant function of key transcriptional regulatory factors that normally control skeletal cell differentiation. We focused our studies on two families of transcription factors (the runt homology domain family and the homeodomain family) that have been shown to regulate the expression of multiple genes selectively expressed by cells of skeletal lineages (12–18). We examined the regulation of the human *bsp* promoter in several well-characterized human breast cancer cell lines previously demonstrated to express BSP at both the mRNA and protein level (1, 3). Deletion analyses were used to identify important regions within the promoter structure that imparted expression of the *bsp* gene in the breast cancer cells. More focused studies then examined the contribution of specific *cis*-acting elements and factors previously demonstrated to regulate *bsp* expression in skeletal lineage cells. The runt homology domain transcription factor Runx2 (AML3/CBFA1/PEBP2 $\alpha$ A) was found to be ectopically expressed in human breast cancer cells and acts as a positive regulator of *bsp* expression. In addition, the homeobox factor *Msx2* was found to regulate *bsp* in these cells as well. Consistent with our results, previous reports have found *Msx2* to be expressed in developing and adult mammary tissues (19). Although both factors are involved in the regulation of several skeletal related genes in osteoblast cells, the demonstration that Runx2 is expressed in metastatic human breast cancer cells may have broader significance because the ectopic or aberrant expression of human Runx/AML family members has been implicated in the malignant transformation of many other cell types (20–22).

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<sup>3</sup> The abbreviations used are: BSP, bone sialoprotein; HMEC, human mammary epithelial cell; EMSA, electromobility shift assay; OC, osteocalcin.

**MATERIALS AND METHODS**

**Cell Cultures.** The MDA-MB-231 and MCF-7 cell lines were obtained from American Tissue Culture Collection. The LCC15-MB was obtained from Dr. Erik W. Thompson (Victoria, Australia) (23). The normal HMEC was obtained from Clonetics, Inc. (Walkersville, MD). All cell lines were cultured using standard techniques in suggested media conditions. All media were obtained from (Life Technologies, Inc., Rockville, MD).

**Nuclear Extracts and EMSAs.** Nuclear extracts were prepared as described previously (15, 24). Aliquots of the supernatant containing nuclear proteins were quick frozen and stored at  $-80^{\circ}\text{C}$ . Protein concentrations were determined using the Bio-Rad (Bradford) protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Oligonucleotides used for EMSAs were end labeled using  $[^{32}\text{P}]\gamma\text{dATP}$  and T4 polynucleotide kinase. Electromobility shift reactions and antibody super shifts were carried out as described previously (15, 24). Reaction products were loaded onto a 5% polyacrylamide gel in  $0.5\times$  Tris-borate EDTA buffer and run for 1.5 h at 200 v. Gels were dried and subsequently visualized on X-Omat radiographic film (Eastman Kodak, Rochester, NY). Oligonucleotides used for the gel shift analysis include human *bsp* Runx site, GAACGTGGAT-TCTACCAGAAACCCCAA; *oc* Runx site, GCTGCAGTCACCAAC-CACAGATCCTTTGG; human *bsp* site A, GCATTGTGGAATTTAATT-GAAGGTTAGAT; and human *bsp* mutant site A, GCATTGTGGAATgactgacAAGGTTAGAT. All sequences designated in the 5' to 3' orientation with mutated nucleotides indicated in lowercase letters.

**Generation of Human BSP Promoter Expression Constructs.** The promoter-less plasmid pGL3 Basic (Promega Corp., Madison, WI) was used for cloning the different promoter fragments obtained from the human *bsp* gene at the 5' end of the firefly luciferase gene. The longest *bsp* promoter fragment considered in this study as the full-length promoter ( $-2180$  to  $+88$ ) was created by inserting a previously isolated human 2.1-kb genomic fragment into the pGL3 basic vector. The different *bsp* promoter deletion constructs ( $-43$ ), ( $-110$ ), ( $-350$ ), and ( $-435$ ) were then generated by the use of the full-length construct as template for PCR with the forward primers 5'-GAAGGCAAA-GAAGAGTTTATAG-3' (nucleotides  $-43$  to  $-22$ ), 5'-TGGGGGTTTTCTG-GTGAGAA-3' (nucleotides  $-108$  to  $-89$ ), 5'-AGCAAGCTTTCCTTTC-TCG-3' (nucleotides  $-348$  to  $-328$ ), 5'-TTGATGGTTCATCTG CTC-TTT-3' (nucleotides  $-433$  to  $-412$ ), respectively, and the common reverse primer 5'-TCCTCTGCCCTCTCAC-3' (nucleotides  $+20$  to  $+5$ ). PCR products of various lengths were first cloned into pNoTA/T7 shuttle vector using prime PCR Cloner cloning system (5'-3', Inc., Boulder, CO) as specified by the manufacturer. The inserts were excised with *Xho*I and *Mlu*I restriction endonucleases and ligated into the similarly digested pGL3 basic plasmid using a DNA ligation kit (Takara Biochemical, Inc., Berkeley, CA). All constructs were verified by DNA sequencing. pRL-TK (Promega Corp.), a plasmid containing the herpes simplex virus thymidine kinase promoter region upstream the cDNA encoding *Renilla* luciferase, was used as an internal control reporter vector.

**Cloning of MSX2 and DLX5 Expression Constructs.** The following primers (5'-CGGGATCCATGGCTTCTCCGAC-3' and 5'-GGCTCGAGT-TAGGATACATGGATG-3' for *Msx-2*; 5'-CGGGATCCATGACAGGAGT-GTTTG-3' and GGCTCGAGCTAATAAAGCGTCCCG-3' for *Dlx-5*) were used to amplify the previously described cDNAs (17) of *Msx-2* (807 bp, containing amino acids 1-267) and *Dlx-5* (870 bp, containing amino acids 1-289) using Pwo DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The PCR-amplified products were double digested with *Bam*HI-*Xho*I enzymes, gel purified with the Zymoclean DNA cleaning system (Zymo Research, Inc.) and ligated into similarly digested pcDNA3.1/HisC vector (Invitrogen, Carlsbad, CA). The in-frame ligation positive clones for *Msx-2* and *Dlx-5* were confirmed by sequencing.

**Transfections and Expression Assays.** DNA constructs were transfected into the respective cell types using the Lipofectamine reagent (Life Technologies, Inc.) as described previously (15, 24). Unless otherwise noted in the text, all transfections were carried out using  $1\ \mu\text{g}$  of plasmid/well in 6-well dishes. All samples were performed in triplicate in a minimum of three independent experiments. All transfections were internally normalized to a thymidine kinase-driven *Renilla* luciferase to standardize for variations in transfection efficiencies between wells. Cells were harvested 48 h posttransfection in

passive lysis buffer (Promega Corp.) and luciferase values obtained using the Promega dual luciferase assay kit and samples read in a luminometer.

**RESULTS**

**Relative Expression Levels of the Human *bsp* Promoter.** We initially tested by transient transfection assay the full-length ( $-2100$ ) human *bsp* promoter activity to confirm promoter fidelity to the previously reported expression levels in the cell lines used in our studies (Fig. 1A). The *bsp* promoter activity was highest in the osteoblastic UMR-106 cells and expressed at extremely low to undetectable levels in the normal HMECs. *bsp* promoter activity in the MDA-MB-231 and LCC15-MB cancer cells was 20 and 50%, respectively, of the activity in the UMR-106 but many fold in excess to the normal mammary epithelia cells. The differing levels of activity in the LCC15-MB cells in comparison with the MDA-MB-231 cells is relevant because the former cell line was derived from a bone metastasis, whereas the latter line was from a primary lesion. In this context, recent reports have demonstrated that bone metastases express higher BSP levels than visceral metastases and primary lesions in breast cancer patients, thus demonstrating a selectivity of expression (7).

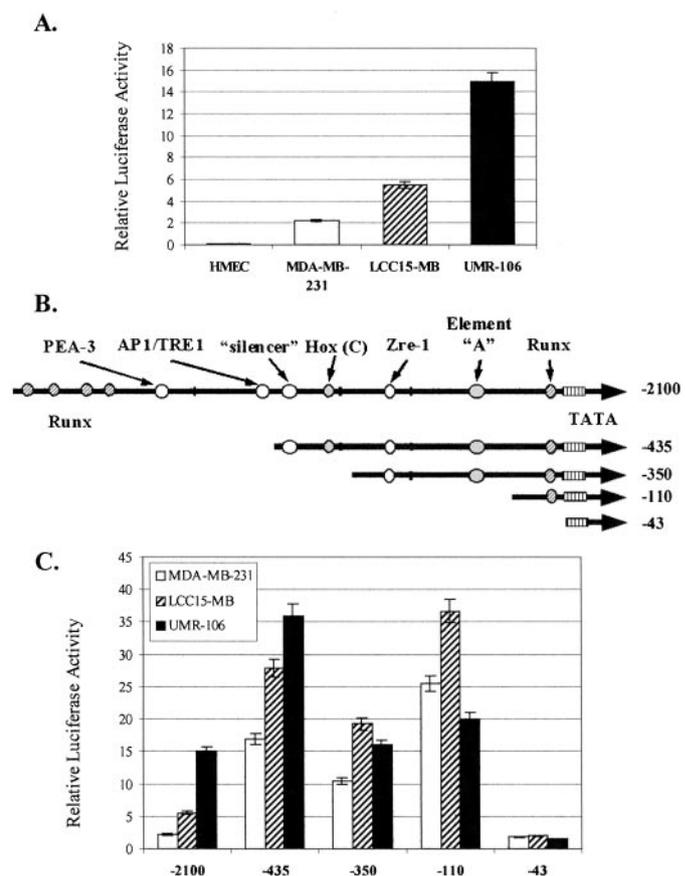


Fig. 1. Comparison of relative BSP promoter activities between normal HMECs the two breast adenocarcinoma lines, MDA-MB-231 and LCC15-MB, respectively, and the osteoblast line UMR-106 (A). Relative activity of the BSP promoter was determined by transient transfection into the respective cell lines and quantification of BSP promoter-linked firefly luciferase activity. Graphical representation of the individual BSP promoter deletion constructs relative to the full-length ( $-2100$ ) human BSP promoter. Elements identified are based on nucleotide homologies to consensus binding elements (B). Deletion analyses of the human BSP promoter. Relative activity of the deletions of the BSP promoter as determined by transient transfection into the respective breast cancer cell lines indicated. All values are relative luciferase activity and are normalized to internal thymidine kinase-driven *Renilla* luciferase controls. Deletion constructs are as detailed in the diagrammatic representation of the human BSP promoter. Individual cell lines tested are as indicated in the figure. All values represent a minimum of three independent experiments each with an  $n = 3$  (C).

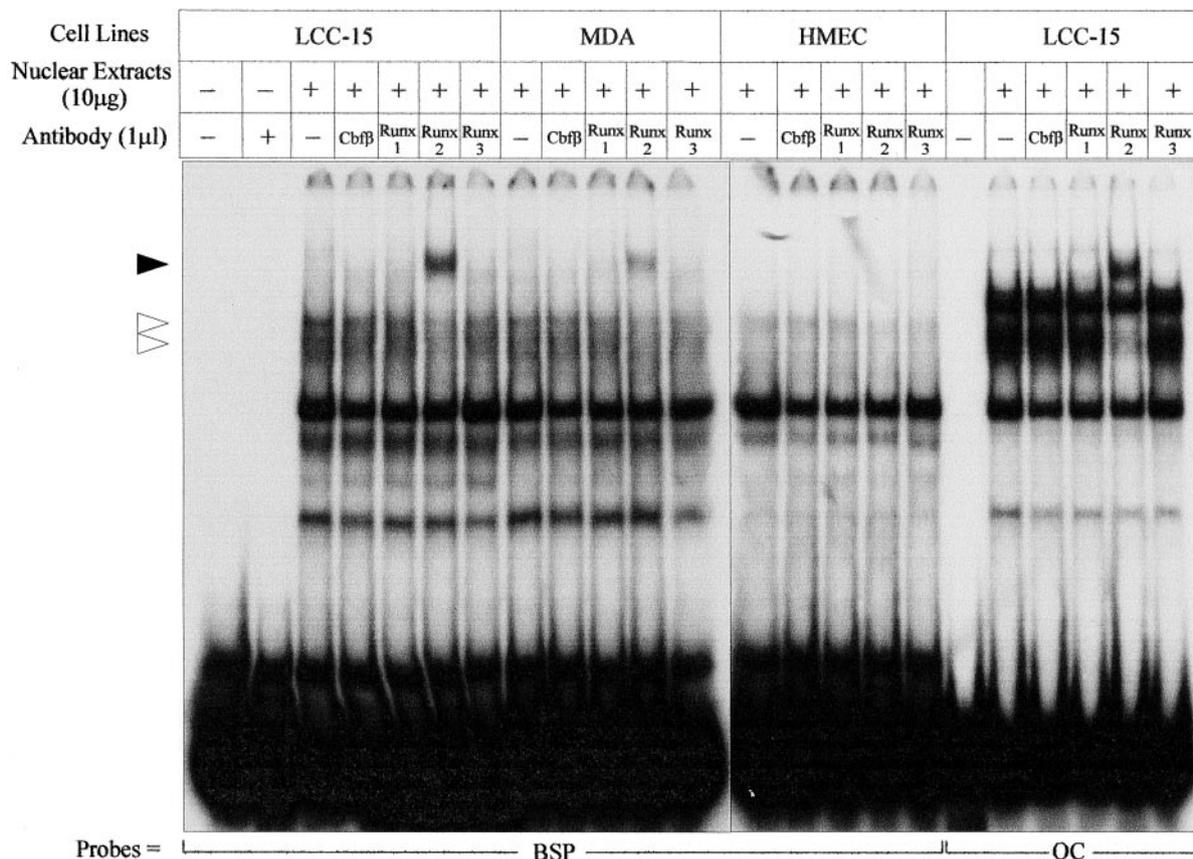


Fig. 2. EMSA of nuclear protein binding to the putative Runx-type *cis*-acting element. Nuclear extracts used are from the respective cell lines indicated at the top of the figure. Specific antibodies used in each lane are as indicated at the top of the figure. Probe designations are indicated at the bottom of the figure and include BSP = wild-type Runx element corresponding to site located in the proximal -110 bp of the human BSP promoter, OC = previously described Runx2 element of the OC promoter demonstrated to bind all members of the Runx family. > indicates the positions of the specific binding complexes, and ▲ indicates the position of the band supershifted by the Runx2-specific antisera. Note that only the Runx2-specific antisera produces a supershift of the retarded complex in the LCC15-MB (designated LCC-15) and MDA-MB-231 (MDA) cells using the BSP promoter sequence. OC promoter sequence complex also supershifted only with the Runx2-specific antisera.

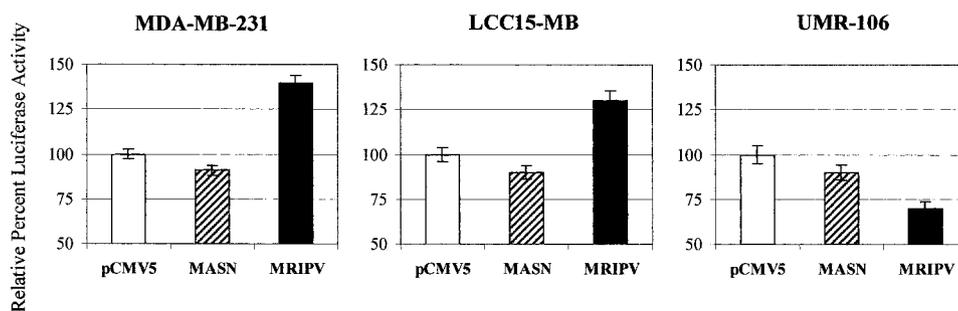
Thus, our results demonstrate that the *bsp* promoter reflects the previously reported relative endogenous *bsp* expression levels.

**Basal *bsp* Promoter (-110 bp) Retains High Level of Activity in Cancer Cells.** Subsequent analysis of the human *bsp* promoter was designed to identify important regulatory regions within the promoter structure that are functionally linked to activity in breast cancer cells. For these studies, a series of deletions was generated in which distal portions of the promoter were sequentially removed (Fig. 1, B and C). The deletion analysis demonstrated that the majority of *cis*-elements within the human *bsp* promoter acted as repressors of *bsp* promoter activity in human breast cancer cells (*i.e.*, the -110-bp construct displayed the highest activity). This result is in contrast to the expression pattern in skeletal-related osteoblast cells (UMR-106) in which the -435 construct displayed the highest levels of expression (Fig. 1C, UMR-106 cell data). The high level of activity displayed by the

-110-bp construct in the breast cancer cells was common to all of the cancer cell lines tested. Analysis of the sequence in this region for homologies to previously identified *cis*-acting elements identified a putative Runx DNA binding motif. Additional analyses of this element are detailed below.

**A Regulatory Element Mediates *bsp* Activity in Breast Cancer Cells.** On the basis of the observation that a putative Runx DNA binding sequence was contained in the -110-bp human *bsp* promoter, we examined the binding of this element by members of the Runx family of factors by electromobility shift and supershift with specific antibodies to each of the family members and their heteromeric partner Cbfb. These studies demonstrate that Runx2 is the major component of the complex that interacts with this proximal element (Fig. 2) based on the ability to supershift a specific band with the Runx2-specific antibody. In contrast, extracts from control HMECs

Fig. 3. Transient transfection analyses of relative *Bsp* promoter activities in response to overexpression of the two isoforms of Runx2 (Type I, MRIPV and Type II, MASN). The promoter-reporter construct used corresponds to the -2100 full-length human BSP promoter construct. All values represent relative BSP promoter-driven firefly luciferase activity with individual samples normalized to internal thymidine kinase Renilla luciferase controls. All values represent a minimum of three independent experiments each with a *n* = 3. Individual cell types are as indicated in the figure. Values are presented as a percentage of BSP promoter activity relative to the control transfection with empty vector (pCMV5).



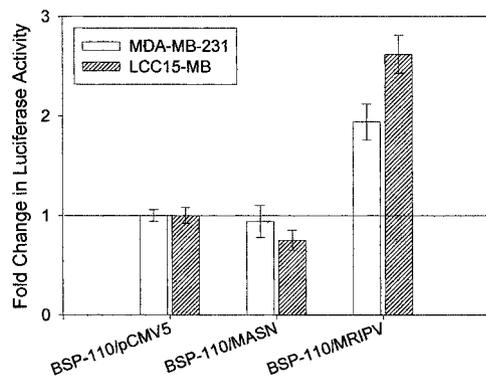


Fig. 4. Transient transfection analyses of relative BSP promoter activities in response to overexpression of the two isoforms of Runx2 (Type I, MRIPV and Type II, MASN). Promoter-reporter construct used corresponds to the -110 deletion construct graphically depicted in Fig. 1B. All values represent relative firefly luciferase activity with individual samples normalized to internal thymidine kinase-driven Renilla luciferase controls. All values are presented as fold change relative to control pCMV5 empty vector cotransfection and represent a minimum of three independent experiments each with an *n* = 3.

(Clontech, Inc.) showed no specific complex formation or complexes that supershifted with any of the antibodies tested (Fig. 2). The observation that only Runx2 is present in these cells was additionally confirmed using the consensus Runx element from the *oc* promoter (right panel of Fig. 2). This element has been used as the prototypical Runx binding element and has been demonstrated to bind all members of the Runx family (15). Extracts from the LCC15-MB cells displayed a supershifted complex only with the Runx2-specific antibody consistent with the shift seen when using the element from the *bsp* gene. These observations demonstrate that in human breast cancer cells but not normal breast cells Runx2 is expressed and specifically binds the element identified in the -110-bp construct and suggest that Runx2 is the primary Runx family member active in breast cancer cells.

To additionally demonstrate the regulation of *bsp* by Runx2, we carried out a series of experiments in which the two isoforms of Runx2 were overexpressed and their effect on *bsp* promoter-reporter expression was determined (Fig. 3). In the osteoblastic UMR-106 cells, both of the Runx2 isoforms (MASN and MRIPV) inhibited human *bsp* promoter (-2100 bp) activity consistent with our previously published data using the Gallus *bsp* promoter (Fig. 3; Ref. 15). In the breast cancer cells, the Runx2 isoforms displayed a unique divergence in regulation of *bsp* promoter activity. These two isoforms are denoted according to the NH<sub>2</sub>-terminal amino acids with the type II isoform-denoted MASN and the Type I as MRIPV (25, 26). Both isoforms are expressed in osteoblasts. Type I (MRIPV) was originally identified in T cells and has a broader expression profile in nonbone tissues (27, 28). In these studies, the MASN isoform repressed *bsp* promoter activity consistent with our previous results in osteoblasts, whereas the MRIPV isoform enhanced full-length human *bsp* promoter activity in both the MDA-MB-231 and LCC15-MB cells (25 and 20% respectively, Fig. 3). This effect was greater when the -110-bp *bsp* promoter construct was used in an equivalent set of experiments (Fig. 4). This *bsp* promoter construct was also inhibited by the MASN isoform, but promoter activity was enhanced by the MRIPV isoform (150–250%) over control levels in the MDA-MB-231 and LCC15-MB cells, respectively. Taken together, the data on the Runx family of transcription factors demonstrates that Runx2 is expressed in the two cancer lines MDA-MB-231 and LCC15-MB but not the normal mammary cell line HMEC. Furthermore, our data demonstrates one of two Runx2 isoforms, the MRIPV isoform, acts as a positive regulator of *bsp* promoter activity and primarily functions through the most proximal Runx binding element.

**Role of the Osteoblast-related *cis*-acting Enhancer Element in the Ectopic Regulation of *bsp*.** In our previous studies, we have identified a *cis*-acting element in the *bsp* promoter that interacts with regulatory factors in a tissue-specific manner (24). These studies additionally showed that this distal element (denoted element A) acts as a tissue-specific enhancer of *bsp* expression in skeletal cells. On the basis of these previous results, we examined both the ability of nuclear extracts to bind this element in cancer cells and the effect of site directed mutagenesis on *bsp* promoter activity.

Analysis of nuclear factor binding at this element was compared between the MDA-MB-231 and LCC15-MB cancer cell lines to the HMEC normal mammary epithelial cells (Fig. 5B). Factor binding at the A element was clearly detectable in both the cancer cell lines and the normal mammary cells. In the case of the A element, a very complex pattern of multiple bands was seen, and more intense complex formation was observable in the LCC15-MB and MDA-MB-231

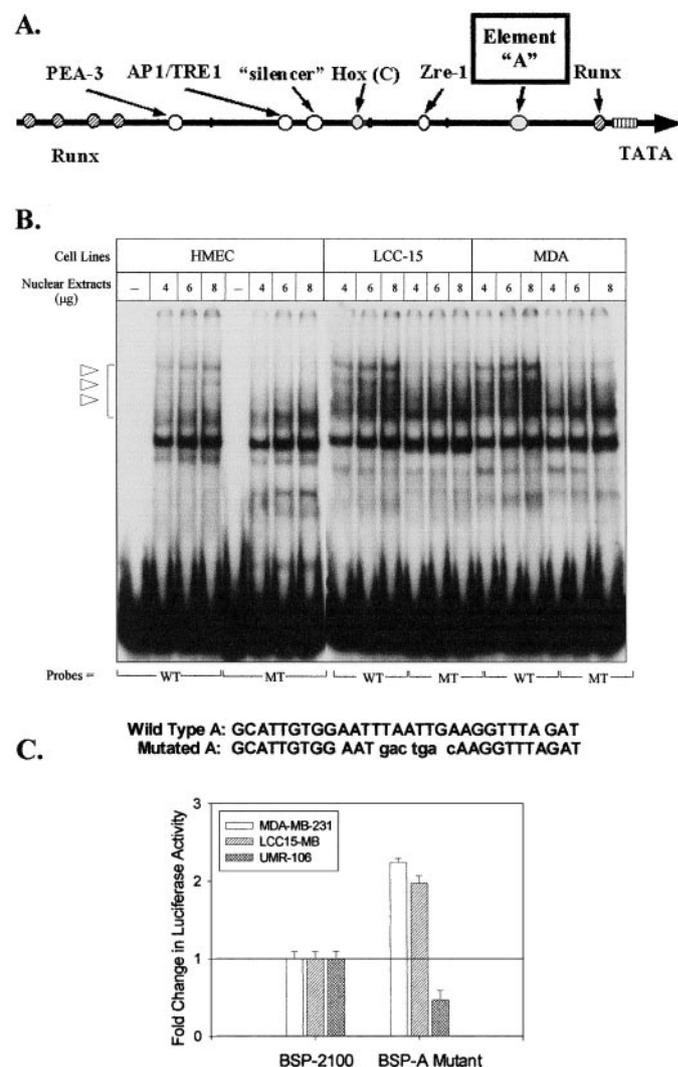


Fig. 5. Diagrammatic representation of the -2100 BSP promoter structure (A). EMSA of protein binding to the *cis*-acting element designated A. Nuclear extracts are from the respective cell lines as indicated at the top of the figure. Total nuclear extract protein content as indicated.  $\triangleright$  indicates positions of specific binding complexes. Probe designations: WT, wild-type element; MT, mutated sequence equivalent to the site directed mutagenesis of the A element. Mutagenesis of the A element leads to increased BSP promoter activity in the human breast cancer cell lines in contrast to the UMR-106 osteoblast-like cells. All values represent relative firefly luciferase activity with individual samples normalized to internal thymidine kinase-driven Renilla luciferase controls. All values represent a minimum of three independent experiments each with an *n* = 3.

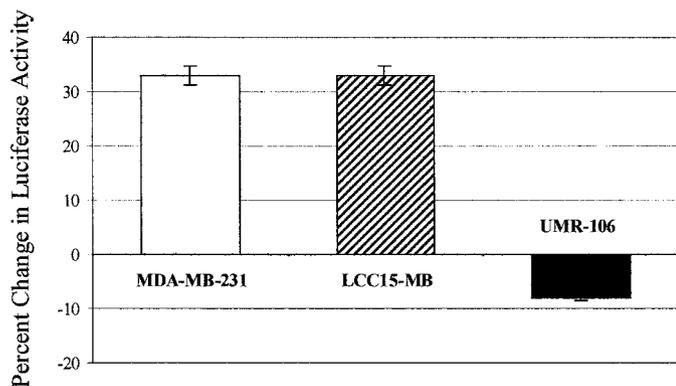


Fig. 6. Transient transfection analyses of relative BSP promoter activities in response to overexpression of the homeodomain factor Dlx5. Promoter-reporter construct used corresponds to the full-length human BSP promoter (-2100 construct). All values are presented as percent change in activity relative to the corresponding control value of the pCMV5 empty vector cotransfection. All values are normalized to internal thymidine kinase-driven Renilla luciferase controls and represent a minimum of three independent experiments each with an  $n = 3$ .

cell lines relative to the HMEC cells, but in each case, similar mobility complexes were formed. Site-directed mutagenesis of the element A lead to increased *bsp* promoter activity in transient transfection assays in the breast cancer cell lines (Fig. 5C). These results demonstrate that this element acts as a repressor of *bsp* expression in these cancer cell lines. Taken together, this data demonstrates that the *cis*-acting element (denoted A) is involved in the regulation of *bsp* expression in cancer cells and functionally represses *bsp* activity. Furthermore, nuclear factor binding at this element does not correlate with *bsp* expression in these cells. Thus, the data supports a functional role for this element, but the mechanism by which this regulation occurs appears to be indirect.

**Regulation of *bsp* by Homeodomain Factors.** The regulatory function of two homeodomain factors, Dlx5 and Msx2, previously implicated in the regulation of bone-specific genes within osteogenic cells was tested in these cancer cells. Msx2 is expressed during fetal and postnatal mammary gland development (19) and is a known regulator of a number of osteoblast-related genes (14, 17, 27, 28). Although Dlx5 is not expressed in breast tissues, Dlx5 has also been demonstrated to be an important regulator of a number of skeletal genes and to regulate osteoblast differentiation (13, 16, 29). In the cancer cell lines MDA-MB-231 and LCC15-MB, Dlx5 overexpression leads to an increase in *bsp* promoter activity (Fig. 6). Overexpression of Msx2, on the other hand, leads to a dramatic reduction (50%) in *bsp* expression in all of the cell lines tested, including the osteoblast cells (Fig. 7). This is consistent with our other recent data on *bsp* regulation in skeletal cells, demonstrating Msx2 represses *bsp* promoter activity (30).

There have been several recent reports demonstrating that Msx2 and Dlx5 act as antagonists in the regulation of skeletal genes, specifically OC. We have recently observed that *bsp* is also regulated through the interactions of Msx2 and Dlx5 in osteoblastic cells in a mechanism independent of DNA binding (Fig. 7 and unpublished observations). These observations lead us to examine if the Msx2 inhibition of *bsp* expression in human breast cancer cells could be antagonized by competition with increasing levels of Dlx5 in transient transfection assays. As observed in the osteoblastic UMR-106 cells, increasing levels of Dlx5 construct expressed in the LCC15-MB and MDA-MB-231 cells relieves the repression of *bsp* expression by Msx2 (Fig. 7, bottom panel). One observation of note is the difference in relative ratio of Dlx5 to Msx2 necessary to relieve inhibition in the UMR-106 *versus* cancer cell lines. The UMR-106 cells respond to

much lower expression levels of Dlx5, suggesting that the expression of Msx2 is lower in these cells or the level of endogenous antagonist (Dlx5 in bone cells) is higher.

**DISCUSSION**

We have postulated that the partial mimicry of an osteoblastic phenotype is an important component of the preferential metastasis of cancers to skeletal sites. To achieve an osteoblast like phenotype we hypothesize that cancer cells are likely to express one or more transcriptional regulators commonly found in skeletal cells. To identify possible regulators of metastasis associated skeletal preference, we have examined the molecular regulation of *bsp* expression in human breast cancer cell lines with known metastatic potentials. *bsp* was chosen as the focus of these studies based on the extensive literature, demonstrating a correlation between BSP expression and skeletal metastasis in several different types of malignancies (4, 7-9, 31). These studies identify two regulatory systems involved in regulating *bsp* levels in human breast cancer cells, the runt homology domain

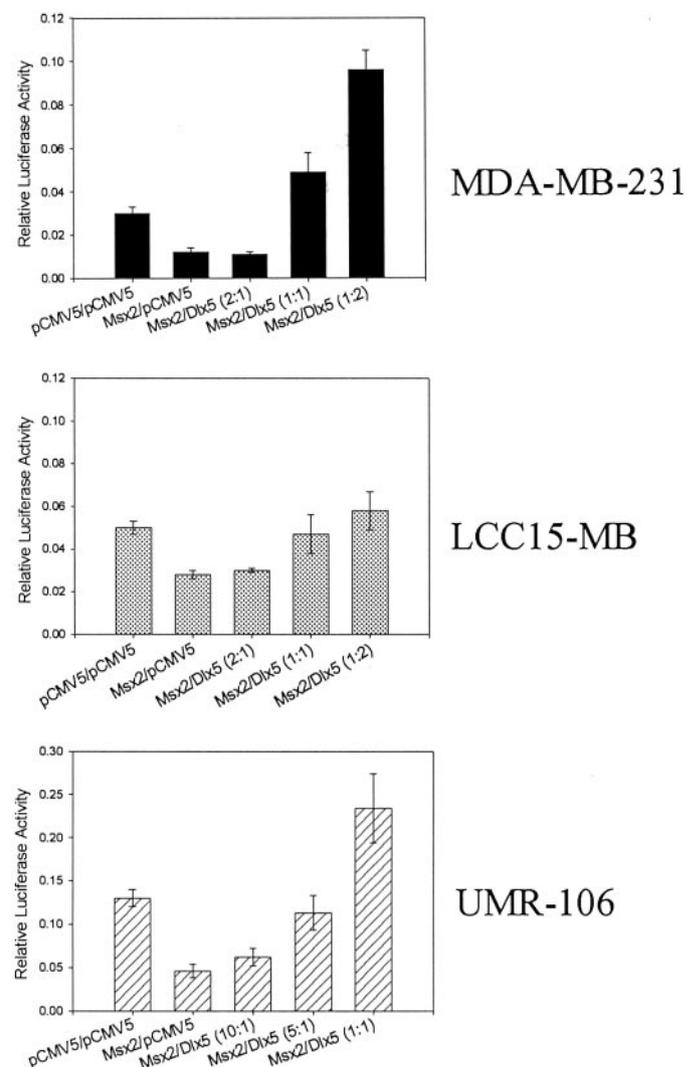


Fig. 7. Transient transfection analyses of relative BSP promoter activities in response to overexpression of the homeodomain factors Msx2 and Dlx5. Relative ratio of Msx2 to Dlx construct used is indicated at the bottom of each panel. Promoter-reporter construct used corresponds to the full-length human BSP promoter (-2100 construct). All values represent relative firefly luciferase activity with individual samples normalized to internal thymidine kinase-driven Renilla luciferase controls. All values represent a minimum of three independent experiments each with an  $n = 3$ .

transcription factor Runx2 (AML3/CBFA1/PEBP2 $\alpha$ A) and the homeodomain factor Msx2. Both of these regulators have well-described roles in osteoblast differentiation and act as central regulators of multiple osteoblast related genes.

The most significant observations of these studies relate to the finding that the runt homology domain transcription factor Runx2 is ectopically expressed in human breast cancer cells (MDA-MB-231 and LCC15-MB) but not normal human mammary cells (HMEC; Fig. 2). In addition, we are able to demonstrate that the MRIPV isoform of Runx2 functions as a positive regulator of *bsp* promoter activity in these cancer cells (Figs. 3 and 4). The significance of this observation pertains to two areas of consideration: first, the centralized role Runx2 plays in osteoblast differentiation and osteoblast-related gene expression; and second, the recognized association of translocations in Runx family members in a variety of cancers.

The importance of Runx2 in skeletal differentiation is most clearly demonstrated by the Runx2 knockout mice. These mice display a complete absence of bone formation because of a lack of differentiated osteoblasts (31). In addition to a central role in osteoblast differentiation, Runx2 directly regulates many osteoblast-related genes (e.g., osteopontin, OC, BSP, MMP-9 and 13, PTHrP, and VEGF; Refs. 15, 18, 25, 32–34). Many of these genes are also preferentially expressed in metastatic cancer cells (5, 35–42). Thus, based on the correlation between known regulatory roles of Runx2 in osteoblast biology and the variety of genes associated with breast cancer metastasis, including *bsp* specifically, we believe that Runx2 is an active regulator of the larger metastatic process consistent with our hypothesis of osteomimicry.

The Runx or CBF family is rapidly being recognized as a family of transcriptional regulators critically important in the differentiation of hematopoietic cells and bone development. As to be expected, disruptions of these key factors have also been identified in a growing number of disease states. For example, genes encoding the Runx1/AML1/CBF $\beta$  transcriptional complex are the most common targets of chromosomal rearrangements in human leukemia (23, 43–45). The Runx2 (Cbf1, Pebp2 $\alpha$ A, AML3) gene has also been previously identified as a frequent target for transcriptional activation by retroviral insertion in T-cell lymphomas in a CD2-MYC transgenic mouse model (20, 21, 27). Furthermore, it has recently been demonstrated that overexpression of the MRIPV Runx2 isoform in the thymus perturbs T-cell development, leads to development of spontaneous lymphomas, and is strongly synergistic with Myc (28). In a separate set of studies, it has been demonstrated that Runx2 is expressed in metastatic prostate cancer cells, PC3 and DU145, and regulates the expression of *oc* in these cells (46). Thus, the association of Runx family members in general and Runx2, in particular, with carcinomas is not restricted to breast cancers.

Our laboratory recently identified a *cis*-acting element (element A) that confers tissue-specific expression to *bsp* in osteoblasts and mature chondrocytes (24). In contrast to the results of our skeletal cell studies, the data from these studies demonstrate that this same element acts in an inhibitory role in these cancer cells (Fig. 5). This function would be consistent with the role of this element in directing the tissue-restricted expression of *bsp*.

We have recently observed that in skeletal cells, Msx2 represses *bsp* expression but that this effect can be de-repressed by increasing Dlx5 levels (Fig. 7, UMR-106 data, and unpublished observations). Regulation through the interaction of Msx2 and Dlx5 has been previously reported for the *oc* promoter and determined to occur independent of direct DNA binding (17). Thus, one mechanism of homeodomain factor regulation of *bsp* in human breast cancers may involve the de-repression of Msx2 inhibition of *bsp* promoter activity by a second interacting homeodomain factor. We have observed that Dlx5

can relieve the inhibition of *bsp* promoter activity by Msx2 in cancer cells (Fig. 7), but additional studies will be needed to identify the specific homeodomain factor interacting with Msx2.

In summary, we have identified two transcriptional regulators of *bsp* expression in human breast cancer cells, Runx2 and Msx2. Each of these factors has well-described roles in regulating osteoblast cellular differentiation and functions to directly regulate many osteoblast-associated genes. The finding that the factor Runx2 is expressed in human breast cancer cell lines and not normal mammary controls is of particular note given the known association of other members of this family of factors with a variety of cancers in addition to the well-described role of Runx2 in directing osteoblast differentiation and regulating osteoblast-associated gene expression. Taken together, this data supports our hypothesis that the osteomimicry displayed by human breast cancer cells is the result of the expression of one or more osteoblast associated factors.

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## Osteoblast-related Transcription Factors Runx2 (Cbfa1/AML3) and MSX2 Mediate the Expression of Bone Sialoprotein in Human Metastatic Breast Cancer Cells

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